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Patentanmeldung Nr. Patent application No. Demande de brevet n°

04090175.3

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

R C van Dijk



Anmeldung Nr:
Application no.: 04090175.3
Demande no:

Anmeldetag:
Date of filing: 06.05.04
Date de dépôt:

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Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Methods and nucleic acids for the improved detection of colon cell proliferative disorders

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s)
revendiquée(s)

Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

EP/27.02.04/EP 04090072

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12Q1/68

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of
filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HU IE IT LU MC NL
PL PT RO SE SI SK TR LI

Methods and nucleic acids for the improved detection of colon cell proliferative disorders.

Introduction

In the United States the annual incidence of colorectal cancer is approximately 150,000, with 56,600 individuals dying from colorectal cancer each year. The lifetime risk of colorectal cancer in the general population is about 5 to 6 percent. Despite intensive efforts in recent years in screening and early detection of colon cancer, until today most cases are diagnosed in an advanced stage with regional or distant metastasis. While the therapeutic options include surgery and adjuvant or palliative chemotherapy, most patients die from progression of their cancer within a few months. Identifying the molecular changes that underly the development of colon cancer may help to develop new monitoring, screening, diagnostic and therapeutic options that could improve the overall poor prognosis of these patients.

The current model of colorectal pathogenesis favours a stepwise progression of adenomas which includes the development of dysplasia and finally signs of invasive cancer. The molecular changes underlying this adenoma-carcinoma sequence include genetic and epigenetic alterations of tumor suppressor genes (APC, p53, DCC), the activation of oncogenes (K-ras) and the inactivation of DNA mismatch repair genes. Recently, further molecular changes and genetic defects have been revealed. Thus, activation of the Wnt signalling pathway not only includes mutations of the APC gene, but may also result from β -catenin mutations. Furthermore, alterations in the TGF- β signalling pathway together with its signal transducers SMAD4 and SMAD2 have been linked to the development of colon cancer.

Despite recent progress in the understanding of the pathogenesis of adenomas and carcinomas of the colon and their genetic and molecular changes, the genetic and epigenetic changes underlying the development of metastasis are less well understood. It is, however, generally well accepted that the process of invasion and proteolysis of the extracellular matrix, as well as infiltration of the vascular basement membrane involve adhesive proteins, such as members of the

family of integrin receptors, the cadherins, the immunoglobulin superfamily, the laminin binding protein and the CD44 receptor. Apart from adhesion, the process of metastasis formation also includes the induction and regulation of angiogenesis (VEGF, bFGF), the induction of cell proliferation (EGF, HGF, IGF) and the activation of proteolytic enzymes (MMPs, TIMPs, uPAR), as well as the inhibition of apoptosis (Bcl-2, Bcl-X). More recently other groups have compared the genetic and molecular changes in metastatic lesions to the changes found in primary colorectal cancers. Thus, Kleeff et al. reported the loss of DOC-2, a candidate tumor suppressor gene, both in primary and metastatic colorectal cancer. Furthermore, Zauber et al. reported that in their series of 42 colorectal cancers Ki-ras mutations in the primary cancers were identical in all of the 42 paired primary and synchronous metastatic lesions. Similarly loss of heterozygosity at the APC locus was identical for 39 paired carcinomas and synchronous metastasis. The authors concluded that for Ki-ras and APC genes the genetic changes in metastasis are identical to the primary colorectal cancer. However, other groups have found genetic and molecular changes in metastatic colon cancers, that are not present in the primary cancers. Thus, the development of LOH of chromosome 3p in colorectal metastasis has been reported. In addition, using comparative genomic hybridization several alterations were found in liver metastasis that were unique to metastatic lesions (-9q, -11q, and -17q).

Apart from mutations aberrant methylation of CpG islands has been shown to lead to the transcriptional silencing of certain genes that have been previously linked to the pathogenesis of various cancers. CpG islands are short sequences which are rich in CpG dinucleotides and can usually be found in the 5' region of approximately 50% of all human genes. Methylation of the cytosines in these islands leads to the loss of gene expression and has been reported in the inactivation of the X chromosome and genomic imprinting.

Recently several groups have also analysed the methylation of various genes in colorectal cancer and reported the transcriptional silencing by promoter methylation for p16INK4, p14ARF, p15INK4b, MGMT, hMLH1, GSTP1, DAPK, CDH1, TIMP-3 and

APC among others. Thus apart from mutational inactivation of certain genes, the hypermethylation of these genes also contributes significantly to the pathogenesis of this disease. In recent years several genes that are methylated in colon cancer have been identified by MS-APPCR. This group of genes among others, includes TPEF/HPPI which is frequently methylated in colon cancers and which was independently identified by two different groups using the MS-APPCR method. See for example, Young J, Biden KG, Simms LA, Huggard P, Karamatic R, Eyre HJ, Sutherland GR, Herath N, Barker M, Anderson GJ, Fitzpatrick DR, Ramm GA, Jass JR, Leggett BA. HPPI: a transmembrane protein-encoding gene commonly methylated in colorectal polyps and cancers. *Proc Natl Acad Sci USA* 2001;98:265-270.

Multifactorial approach. Cancer diagnostics has traditionally relied upon the detection of single molecular markers (e.g. gene mutations, elevated PSA levels). Unfortunately, cancer is a disease state in which single markers have typically failed to detect or differentiate many forms of the disease. Thus, assays that recognize only a single marker have been shown to be of limited predictive value. A fundamental aspect of this invention is that methylation based cancer diagnostics and the screening, diagnosis, and therapeutic monitoring of such diseases will provide significant improvements over the state-of-the-art that uses single marker analyses by the use of a selection of multiple markers. The multiplexed analytical approach is particularly well suited for cancer diagnostics since cancer is not a simple disease, this multi-factorial "panel" approach is consistent with the heterogeneous nature of cancer, both cytologically and clinically.

Key to the successful implementation of a panel approach to methylation based diagnostic tests is the design and development of optimized panels of markers that can characterize and distinguish disease states. This patent application describes an efficient and unique panel of genes the methylation analysis of one or a combination of the members of the panel enabling the detection of cell proliferative disorders of the prostate with a particularly high sensitivity, specificity and/or predictive value.

Development of medical tests. Two key evaluative measures of any medical screening or diagnostic test are its sensitivity and specificity, which measure how well the test performs to accurately detect all affected individuals without exception, and without falsely including individuals who do not have the target disease (predictive value). Historically, many diagnostic tests have been criticized due to poor sensitivity and specificity.

A true positive (TP) result is where the test is positive and the condition is present. A false positive (FP) result is where the test is positive but the condition is not present. A true negative (TN) result is where the test is negative and the condition is not present. A false negative (FN) result is where the test is negative but the condition is not present.

Sensitivity = $TP / (TP + FN)$

Specificity = $TN / (FP + TN)$

Predictive value = $TP / (TP + FP)$

Sensitivity is a measure of a test's ability to correctly detect the target disease in an individual being tested. A test having poor sensitivity produces a high rate of false negatives, i.e., individuals who have the disease but are falsely identified as being free of that particular disease. The potential danger of a false negative is that the diseased individual will remain undiagnosed and untreated for some period of time, during which the disease may progress to a later stage wherein treatments, if any, may be less effective. An example of a test that has low sensitivity is a protein-based blood test for HIV. This type of test exhibits poor sensitivity because it fails to detect the presence of the virus until the disease is well established and the virus has invaded the bloodstream in substantial numbers. In contrast, an example of a test that has high sensitivity is viral-load detection using the polymerase chain reaction (PCR). High sensitivity is achieved because this type of test can detect very small quantities of the virus. High sensitivity is particularly important when the consequences of missing a diagnosis are high.

Specificity, on the other hand, is a measure of a test's ability to identify accurately patients who are free of the disease state. A test having poor specificity produces a high rate of false positives, i.e., individuals who are falsely identified as having the disease. A drawback of false positives is that they force patients to undergo unnecessary medical procedures treatments with their attendant risks, emotional and financial stresses, and which could have adverse effects on the patient's health. A feature of diseases which makes it difficult to develop diagnostic tests with high specificity is that disease mechanisms, particularly in cancer, often involve a plurality of genes and proteins. Additionally, certain proteins may be elevated for reasons unrelated to a disease state. An example of a test that has high specificity is a gene-based test that can detect a p53 mutation. Specificity is important when the cost or risk associated with further diagnostic procedures or further medical intervention are very high.

Bisulfite modification of DNA is an art-recognized tool used to assess CpG methylation status. 5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis. Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing, because 5-methylcytosine has the same base pairing behavior as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during, e.g., PCR amplification.

The most frequently used method for analyzing DNA for the presence of 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine whereby, upon subsequent alkaline hydrolysis, cytosine is converted to uracil which corresponds to thymine in its base pairing behavior. Significantly, however, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is

converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridization behavior, can now be detected as the only remaining cytosine using standard, art-recognized molecular biological techniques, for example, by amplification and hybridization, or by sequencing. All of these techniques are based on differential base pairing properties, which can now be fully exploited.

The prior art, in terms of sensitivity, is defined by a method comprising enclosing the DNA to be analyzed in an agarose matrix, thereby preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and replacing all precipitation and purification steps with fast dialysis (Olek A, et al., A modified and improved method for bisulfite based cytosine methylation analysis, *Nucleic Acids Res.* 24:5064-6, 1996). It is thus possible to analyze individual cells for methylation status, illustrating the utility and sensitivity of the method. An overview of art-recognized methods for detecting 5-methylcytosine is provided by Rein, T., et al., *Nucleic Acids Res.*, 26:2255, 1998.

The bisulfite technique, barring few exceptions (e.g., Zeschnigk M, et al., *Eur J Hum Genet.* 5:94-98, 1997), is currently only used in research. In all instances, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment, and either completely sequenced (Olek & Walter, *Nat Genet.* 1997 17:275-6, 1997), subjected to one or more primer extension reactions (Gonzalzo & Jones, *Nucleic Acids Res.*, 25:2529-31, 1997; WO 95/00669; U.S. Patent No. 6,251,594) to analyze individual cytosine positions, or treated by enzymatic digestion (Xiong & Laird, *Nucleic Acids Res.*, 25:2532-4, 1997). Detection by hybridization has also been described in the art (Olek et al., WO 99/28498). Additionally, use of the bisulfite technique for methylation detection with respect to individual genes has been described (Grigg & Clark, *Bioessays*, 16:431-6, 1994; Zeschnigk M, et al., *Hum Mol Genet.*, 6:387-95, 1997; Feil R, et al., *Nucleic Acids Res.*, 22:695-, 1994; Martin V, et al., *Gene*, 157:261-4, 1995; WO 9746705 and WO 9515373).

Bisulfite Methylation Assay Procedures. Various methylation assay procedures are known in the art, and can be used in

conjunction with the present invention. These assays allow for determination of the methylation state of one or a plurality of CpG dinucleotides (e.g., CpG islands) within a DNA sequence. Such assays involve, among other techniques, DNA sequencing of bisulfite-treated DNA, PCR (for sequence-specific amplification), Southern blot analysis, and use of methylation-sensitive restriction enzymes.

For example, genomic sequencing has been simplified for analysis of DNA methylation patterns and 5-methylcytosine distribution by using bisulfite treatment (Frommer et al., *Proc. Natl. Acad. Sci. USA* 89:1827-1831, 1992). Additionally, restriction enzyme digestion of PCR products amplified from bisulfite-converted DNA is used, e.g., the method described by Sadri & Hornsby (*Nucl. Acids Res.* 24:5058-5059, 1996), or COBRA (Combined Bisulfite Restriction Analysis) (Xiong & Laird, *Nucleic Acids Res.* 25:2532-2534, 1997).

COBRA. COBRA analysis is a quantitative methylation assay useful for determining DNA methylation levels at specific gene loci in small amounts of genomic DNA (Xiong & Laird, *Nucleic Acids Res.* 25:2532-2534, 1997). Briefly, restriction enzyme digestion is used to reveal methylation-dependent sequence differences in PCR products of sodium bisulfite-treated DNA. Methylation-dependent sequence differences are first introduced into the genomic DNA by standard bisulfite treatment according to the procedure described by Frommer et al. (*Proc. Natl. Acad. Sci. USA* 89:1827-1831, 1992). PCR amplification of the bisulfite converted DNA is then performed using primers specific for the interested CpG islands, followed by restriction endonuclease digestion, gel electrophoresis, and detection using specific, labeled hybridization probes. Methylation levels in the original DNA sample are represented by the relative amounts of digested and undigested PCR product in a linearly quantitative fashion across a wide spectrum of DNA methylation levels. In addition, this technique can be reliably applied to DNA obtained from microdissected paraffin-embedded tissue samples.

Other assays used in the art include "MethyLight" (a fluorescence-based real-time PCR technique) (Eads et al., *Cancer Res.* 59:2302-2306, 1999), Ms-SNuPE (Methylation-sensitive Single Nucleotide Primer Extension) reactions

(Gonzalzo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997), methylation-specific PCR ("MSP"; Herman et al., *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996; US Patent No. 5,786,146), and methylated CpG island amplification ("MCA"; Toyota et al., *Cancer Res.* 59:2307-12, 1999). These may be used alone or in combination with other of these methods.

MethyLight. The MethyLight assay is a high-throughput quantitative methylation assay that utilizes fluorescence-based real-time PCR (TaqMan•) technology that requires no further manipulations after the PCR step (Eads et al., *Cancer Res.* 59:2302-2306, 1999). Briefly, the MethyLight process begins with a mixed sample of genomic DNA that is converted, in a sodium bisulfite reaction, to a mixed pool of methylation-dependent sequence differences according to standard procedures (the bisulfite process converts unmethylated cytosine residues to uracil). Fluorescence-based PCR is then performed either in an "unbiased" (with primers that do not overlap known CpG methylation sites) PCR reaction, or in a "biased" (with PCR primers that overlap known CpG dinucleotides) reaction. Sequence discrimination can occur either at the level of the amplification process or at the level of the fluorescence detection process, or both.

The MethyLight assay may be used as a quantitative test for methylation patterns in the genomic DNA sample, wherein sequence discrimination occurs at the level of probe hybridization. In this quantitative version, the PCR reaction provides for unbiased amplification in the presence of a fluorescent probe that overlaps a particular putative methylation site. An unbiased control for the amount of input DNA is provided by a reaction in which neither the primers, nor the probe overlap any CpG dinucleotides. Alternatively, a qualitative test for genomic methylation is achieved by probing of the biased PCR pool with either control oligonucleotides that do not "cover" known methylation sites (a fluorescence-based version of the "MSP" technique), or with oligonucleotides covering potential methylation sites.

The MethyLight process can be used with a "TaqMan®" probe in the amplification process. For example, double-stranded genomic DNA is treated with sodium bisulfite and subjected to one of two sets of PCR reactions using TaqMan® probes; e.g.,

with either biased primers and TaqMan® probe, or unbiased primers and TaqMan® probe. The TaqMan® probe is dual-labeled with fluorescent "reporter" and "quencher" molecules, and is designed to be specific for a relatively high GC content region so that it melts out at about 10°C higher temperature in the PCR cycle than the forward or reverse primers. This allows the TaqMan® probe to remain fully hybridized during the PCR annealing/extension step. As the Taq polymerase enzymatically synthesizes a new strand during PCR, it will eventually reach the annealed TaqMan® probe. The Taq polymerase 5' to 3' endonuclease activity will then displace the TaqMan® probe by digesting it to release the fluorescent reporter molecule for quantitative detection of its now unquenched signal using a real-time fluorescent detection system.

Alternatively the MethyLight process can be used with 'Lightcycler' probes. A LightCycler probe is a pair of single-stranded fluorescent-labeled oligonucleotides. The first oligonucleotide probe is labeled at its 3' end with a donor fluorophore dye and the second is labeled at its 5' end with an acceptor fluorophore dyes. The free 3' hydroxyl group of the second probe is blocked with a phosphate group to prevent polymerase mediated extension.

During the annealing step of real-time quantitative PCR, the PCR primers and the LightCycler probes hybridize to their specific target regions causing the donor dye to come into close proximity to the acceptor dye. When the donor dye is excited by light, energy is transferred by Fluorescence Resonance Energy Transfer (FRET) from the donor to the acceptor dye. The energy transfer causes the acceptor dye to emit fluorescence wherein the increase of measured fluorescence signal is directly proportional to the amount of target DNA.

Typical reagents (e.g., as might be found in a typical MethyLight-based kit) for MethyLight analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); TaqMan® and/or LightCycler probes; optimized PCR buffers and deoxynucleotides; and Taq polymerase.

Ms-SNuPE. The Ms-SNuPE technique is a quantitative method for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA, followed by single-

nucleotide primer extension (Gonzalzo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997). Briefly, genomic DNA is reacted with sodium bisulfite to convert unmethylated cytosine to uracil while leaving 5-methylcytosine unchanged. Amplification of the desired target sequence is then performed using PCR primers specific for bisulfite-converted DNA, and the resulting product is isolated and used as a template for methylation analysis at the CpG site(s) of interest. Small amounts of DNA can be analyzed (e.g., microdissected pathology sections), and it avoids utilization of restriction enzymes for determining the methylation status at CpG sites.

Typical reagents (e.g., as might be found in a typical Ms-SNuPE-based kit) for Ms-SNuPE analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); optimized PCR buffers and deoxynucleotides; gel extraction kit; positive control primers; Ms-SNuPE primers for specific gene; reaction buffer (for the Ms-SNuPE reaction); and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kit (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

MSP. MSP (methylation-specific PCR) allows for assessing the methylation status of virtually any group of CpG sites within a CpG island, independent of the use of methylation-sensitive restriction enzymes (Herman et al. *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996; US Patent No. 5,786,146). Briefly, DNA is modified by sodium bisulfite converting all unmethylated, but not methylated cytosines to uracil, and subsequently amplified with primers specific for methylated versus unmethylated DNA. This technique has been described in United States Patent No. 6,265,171 to Herman. The use of methylation status specific primers for the amplification of bisulfite treated DNA allows the differentiation between methylated and unmethylated nucleic acids. MSP primer pairs contain at least one primer which hybridizes to a bisulfite treated CpG dinucleotide. Therefore, the sequence of said primers comprises at least one CpG dinucleotide. MSP primers specific for non-methylated DNA contain a "T" at the 3' position of the C position in the CpG. MSP requires only small quantities of DNA, is sensitive to 0.1%

methyated alleles of a given CpG island locus, and can be performed on DNA extracted from paraffin-embedded samples. Typical reagents (e.g., as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene (or methylation-altered DNA sequence or CpG island), optimized PCR buffers and deoxynucleotides, and specific probes.

Pronounced need in the art. Therefore, in view of the incidence of colon cancer there is a substantial need in the art for the development of molecular markers that could be used for the early detection of colon cell proliferative disorders, in particular colon cancer. Additionally, there is a pronounced need in the art for the development of molecular markers that could be used to provide sensitive, accurate and non-invasive methods (as opposed to, e.g., biopsy) for the diagnosis, prognosis and treatment of colon cell proliferative disorders.

DETAILED DESCRIPTION OF THE INVENTION

For the purposes of the following invention the sensitivity and specificity refer to values calculated by reference to a sample set according to that described in the herein contained examples.

Definitions:

The term "Observed/Expected Ratio" ("O/E Ratio") refers to the frequency of CpG dinucleotides within a particular DNA sequence, and corresponds to the $[\text{number of CpG sites} / (\text{number of C bases} \cdot \text{number of G bases})] \cdot \text{band length for each fragment}$.

The term "CpG island" refers to a contiguous region of genomic DNA that satisfies the criteria of (1) having a frequency of CpG dinucleotides corresponding to an "Observed/Expected Ratio" >0.6 , and (2) having a "GC Content" >0.5 . CpG islands are typically, but not always, between about 0.2 to about 1 kb in length.

The term "methylation state" or "methylation status" refers to the presence or absence of 5-methylcytosine ("5-mCyt") at one or a plurality of CpG dinucleotides within a DNA sequence. Methylation states at one or more particular palindromic CpG

methylation sites (each having two CpG CpG dinucleotide sequences) within a DNA sequence include "unmethylated," "fully-methylated" and "hemi-methylated."

The term "hemi-methylation" or "hemimethylation" refers to the methylation state of a palindromic CpG methylation site, where only a single cytosine in one of the two CpG dinucleotide sequences of the palindromic CpG methylation site is methylated (e.g., 5'-CC^MGG-3' (top strand): 3'-GGCC-5' (bottom strand)).

The term "hypermethylation" refers to the average methylation state corresponding to an *increased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

The term "hypomethylation" refers to the average methylation state corresponding to a *decreased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

The term "microarray" refers broadly to both "DNA microarrays," and "DNA chip(s)," as recognized in the art, encompasses all art-recognized solid supports, and encompasses all methods for affixing nucleic acid molecules thereto or synthesis of nucleic acids thereon.

"Genetic parameters" are mutations and polymorphisms of genes and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

"Epigenetic parameters" are, in particular, cytosine methylations. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analyzed using the described method but which, in turn, correlate with the DNA methylation.

The term "bisulfite reagent" refers to a reagent comprising bisulfite, disulfite, hydrogen sulfite or combinations thereof, useful as disclosed herein to distinguish between methylated and unmethylated CpG dinucleotide sequences.

The term "Methylation assay" refers to any assay for determining the methylation state of one or more CpG dinucleotide sequences within a sequence of DNA.

The term "MS.AP-PCR" (Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction) refers to the art-recognized technology that allows for a global scan of the genome using CG-rich primers to focus on the regions most likely to contain CpG dinucleotides, and described by Gonzalgo et al., *Cancer Research* 57:594-599, 1997.

The term "MethyLight™" refers to the art-recognized fluorescence-based real-time PCR technique described by Eads et al., *Cancer Res.* 59:2302-2306, 1999.

The term "HeavyMethyl" assay, in the embodiment thereof implemented herein, refers to an assay comprising the use of methylation specific *blocking* probes covering CpG positions between the amplification primers.

The term "Ms-SNuPE" (Methylation-sensitive Single Nucleotide Primer Extension) refers to the art-recognized assay described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997.

The term "MSP" (Methylation-specific PCR) refers to the art-recognized methylation assay described by Herman et al. *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996, and by US Patent No. 5,786,146.

The term "COBRA" (Combined Bisulfite Restriction Analysis) refers to the art-recognized methylation assay described by Xiong & Laird, *Nucleic Acids Res.* 25:2532-2534, 1997.

The term "MCA" (Methylated CpG Island Amplification) refers to the methylation assay described by Toyota et al., *Cancer Res.* 59:2307-12, 1999, and in WO 00/26401A1.

The term "hybridization" is to be understood as a bond of an oligonucleotide to a complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure.

"Stringent hybridization conditions," as defined herein, involve hybridizing at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature, or involve the art-recognized equivalent thereof (e.g., conditions in which a hybridization is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at

37°C in a low buffer concentration, and remains stable). Moderately stringent conditions, as defined herein, involve including washing in 3x SSC at 42°C, or the art-recognized equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Guidance regarding such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

Overview:

Despite intensive efforts to improve screening and early detection of colon cell proliferative disorders, most cases are diagnosed in an advanced stage with regional or distant metastasis which are associated with poor survival. The herein described invention discloses epigenetic markers that have novel utility for the analysis of colon cell proliferative disorders combined with sensitive assay methods for the improved detection, classification, treatment and overall prognosis of said disorders. The invention presents improvements over the state of the art in that it provides a means for the detection, classification and prognosis of colon cell proliferative disorders by analysis of a gene panel, with a high sensitivity and specificity. The invention presents further improvements in that the 'gene panel' consists of only 15 genes and/or their regulatory sequences, thereby enabling a highly sensitive and specific but time and cost effective analysis of a limited number of genes and/or their regulatory sequences.

In one aspect, the present invention provides for the use of the bisulfite technique, in combination with one or more methylation assays, for determination of the methylation status of CpG dinucleotide sequences within sequences from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 15. According to the present invention, determination of the methylation status of CpG dinucleotide sequences within sequences from the group

consisting of SEQ ID NO: 1 to SEQ ID NO: 15 has diagnostic and prognostic utility.

GENOMIC SEQUENCES ACCORDING TO SEQ ID NO: 1 to SEQ ID NO: 15, AND TREATED VARIANTS THEREOF ACCORDING TO SEQ ID NO: 16 to SEQ ID NO: 75, WERE DETERMINED TO HAVE UTILITY FOR THE DETECTION, CLASSIFICATION AND/OR TREATMENT OF COLON CELL PROLIFERATIVE DISORDERS.

In one aspect the present invention provides a selection of genetic sequences according to Table 1. One or more of these genes are analysed in the form of a 'gene panel'. This aspect of the invention is further based upon the analysis of methylation levels within one or more genomic sequences taken from the group consisting SEQ ID NO: 1 to SEQ ID NO: 15.

Particular embodiments of the present invention provide a novel application of the analysis of methylation levels and/or patterns within said sequences that enables a precise detection, classification, treatment and overall prognosis of colon cell proliferative disorders. Early detection of colon cell proliferative disorders is directly linked with disease prognosis, and the disclosed method thereby enables the physician and patient to make better and more informed treatment decisions.

Detailed description

The present invention is based upon the analysis of methylation levels within one or more genomic sequences taken from the group according to Table 1 and/or their regulatory sequences. Accordingly, the invention also discloses the genomic sequences of said genes in SEQ ID NO: 1 to SEQ ID NO: 15, according to Table 1. Additional embodiments provide modified variants of SEQ ID NO: 1 to SEQ ID NO: 15, as well as oligonucleotides and/or PNA-oligomers for analysis of cytosine methylation patterns within SEQ ID NO: 1 to SEQ ID NO: 15.

According to the present invention hypermethylation of the genomic sequences according to Table 1 and/or their regulatory sequences is correlated with the presence of colon cell proliferative disorders. The present invention discloses the analysis of methylation within said genes and/or their regulatory sequences in the form of a panel enabling the

improved detection, classification, treatment and overall prognosis of colon cell proliferative disorders.

Hypermethylation of one or more of the the genomic sequences according to Table 1 and/or their regulatory sequences being indicative of the presence of colon carcinoma. Early detection of colon carcinoma increases the chances of patient survival.

An objective of the invention comprises analysis of the methylation state of one or more CpG dinucleotides within at least one of the genomic sequences selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO: 15 and sequences complementary thereto.

Wherein the object of the analysis is the detection of colon cell proliferative disorders it is preferred that the methylation of one or more sequences selected from the group consisting SEQ ID NO: 1 to SEQ ID NO: 15 are analysed.

The disclosed invention provides treated nucleic acids, derived from genomic SEQ ID NO: 1 to SEQ ID NO: 15, wherein the treatment is suitable to convert at least one unmethylated cytosine base of the genomic DNA sequence to uracil or another base that is detectably dissimilar to cytosine in terms of hybridization. The genomic sequences in question may comprise one, or more, consecutive or random methylated CpG positions. Said treatment preferably comprises use of a reagent selected from the group consisting of bisulfite, hydrogen sulfite, disulfite, and combinations thereof .In a preferred embodiment of the invention, the objective comprises analysis of a modified nucleic acid comprising a sequence of at least 16 contiguous nucleotide bases in length of a sequence selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO: 75, wherein said sequence comprises at least one CpG, TpA or CpA dinucleotide and sequences complementary thereto. The sequences of SEQ ID NO: 16 to SEQ ID NO: 75 provide modified versions of the nucleic acid according to SEQ ID NO: 1 to SEQ ID NO: 15, wherein the modification of each genomic sequence results in the synthesis of a nucleic acid having a sequence that is unique and distinct from said genomic sequence as follows. For each sense strand genomic DNA, e.g., SEQ ID NO:1, four converted versions are disclosed. A first version wherein

"C"•is converted to "T," but "CpG" remains "CpG" (i.e., corresponds to case where, for the genomic sequence, all "C" residues of CpG dinucleotide sequences are methylated and are thus not converted); a second version discloses the complement of the disclosed genomic DNA sequence (i.e. antisense strand), wherein "C"•is converted to "T," but "CpG" remains "CpG" (i.e., corresponds to case where, for all "C" residues of CpG dinucleotide sequences are methylated and are thus not converted). The 'upmethylated' converted sequences of SEQ ID NO: 1 to SEQ ID NO: 15 correspond to SEQ ID NO: 16 to SEQ ID NO: 45. A third chemically converted version of each genomic sequences is provided, wherein "C"• is converted to "T" for all "C" residues, including those of "CpG" dinucleotide sequences (i.e., corresponds to case where, for the genomic sequences, all "C" residues of CpG dinucleotide sequences are unmethylated); a final chemically converted version of each sequence, discloses the complement of the disclosed genomic DNA sequence (i.e. antisense strand), wherein "C"• is converted to "T" for all "C" residues, including those of "CpG" dinucleotide sequences (i.e., corresponds to case where, for the complement (antisense strand) of each genomic sequence, all "C" residues of CpG dinucleotide sequences are unmethylated). The 'downmethylated' converted sequences of SEQ ID NO: 1 to SEQ ID NO: 15 correspond to SEQ ID NO: 46 to SEQ ID NO: 75.

In a preferred embodiment, such analysis comprises the use of an oligonucleotide or oligomer for detecting the cytosine methylation state within genomic or pretreated DNA, according to SEQ ID NO: 1 to SEQ ID NO: 75. Said oligonucleotide or oligomer comprising a nucleic acid sequence having a length of at least nine (9) nucleotides which hybridizes, under moderately stringent or stringent conditions (as defined herein above), to a pretreated nucleic acid sequence according to SEQ ID NO: 16 to SEQ ID NO: 75 and/or sequences complementary thereto, or to a genomic sequence according to SEQ ID NO: 1 to SEQ ID NO: 15 and/or sequences complementary thereto.

Thus, the present invention includes nucleic acid molecules (e.g., oligonucleotides and peptide nucleic acid (PNA) molecules (PNA-oligomers)) that hybridize under moderately stringent and/or stringent hybridization conditions

to all or a portion of the sequences SEQ ID NO: 1 to SEQ ID NO: 75, or to the complements thereof. The hybridizing portion of the hybridizing nucleic acids is typically at least 9, 15, 20, 25, 30 or 35 nucleotides in length. However, longer molecules have inventive utility, and are thus within the scope of the present invention.

Preferably, the hybridizing portion of the inventive hybridizing nucleic acids is at least 95%, or at least 98%, or 100% identical to the sequence, or to a portion thereof of SEQ ID NO: 1 to SEQ ID NO: 75, or to the complements thereof.

Hybridizing nucleic acids of the type described herein can be used, for example, as a primer (e.g., a PCR primer), or a diagnostic and/or prognostic probe or primer. Preferably, hybridization of the oligonucleotide probe to a nucleic acid sample is performed under stringent conditions and the probe is 100% identical to the target sequence. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or T_m , which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions.

For target sequences that are related and substantially identical to the corresponding sequence of SEQ ID NO: 1 to SEQ ID NO: 15 (such as allelic variants and SNPs), rather than identical, it is useful to first establish the lowest temperature at which only homologous hybridization occurs with a particular concentration of salt (e.g., SSC or SSPE). Then, assuming that 1% mismatching results in a 1°C decrease in the T_m , the temperature of the final wash in the hybridization reaction is reduced accordingly (for example, if sequences having > 95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in T_m can be between 0.5°C and 1.5°C per 1% mismatch.

Examples of inventive oligonucleotides of length x (in nucleotides), as indicated by polynucleotide positions with reference to, e.g., SEQ ID NO:1, include those corresponding to sets (sense and antisense sets) of consecutively overlapping oligonucleotides of length X , where the oligonucleotides within each consecutively overlapping set (corresponding to a given X

value) are defined as the finite set of Z oligonucleotides from nucleotide positions:

n to (n + (X-1));

where n=1, 2, 3,...(Y-(X-1));

where Y equals the length (nucleotides or base pairs);

where X equals the common length (in nucleotides) of each oligonucleotide in the set (e.g., X=20 for a set of consecutively overlapping 20-mers); and where the number (Z) of consecutively overlapping oligomers of length X for a given SEQ ID NO of length Y is equal to Y-(X-1).

Preferably, the set is limited to those oligomers that comprise at least one CpG, TpG or CpA dinucleotide.

The present invention encompasses, for each of SEQ ID NO: 1 to SEQ ID NO: 75 (sense and antisense), multiple consecutively overlapping sets of oligonucleotides or modified oligonucleotides of length X, where, e.g., X= 9, 10, 17, 20, 22, 23, 25, 27, 30 or 35 nucleotides.

The oligonucleotides or oligomers according to the present invention constitute effective tools useful to ascertain genetic and epigenetic parameters of the genomic sequence corresponding to SEQ ID NO: 1 to SEQ ID NO: 15. Preferred sets of such oligonucleotides or modified oligonucleotides of length X are those consecutively overlapping sets of oligomers corresponding to SEQ ID NO: 1 to SEQ ID NO: 75 (and to the complements thereof). Preferably, said oligomers comprise at least one CpG, TpG or CpA dinucleotide.

Particularly preferred oligonucleotides or oligomers according to the present invention are those in which the cytosine of the CpG dinucleotide (or of the corresponding converted TpG or CpA dinucleotide) sequences is within the middle third of the oligonucleotide; that is, where the oligonucleotide is, for example, 13 bases in length, the CpG, TpG or CpA dinucleotide is positioned within the fifth to ninth nucleotide from the 5'-end.

The oligonucleotides of the invention can also be modified by chemically linking the oligonucleotide to one or more moieties or conjugates to enhance the activity, stability or

detection of the oligonucleotide. Such moieties or conjugates include chromophores, fluorophors, lipids such as cholesterol, cholic acid, thioether, aliphatic chains, phospholipids, polyamines, polyethylene glycol (PEG), palmityl moieties, and others as disclosed in, for example, United States Patent Numbers 5,514,758, 5,565,552, 5,567,810, 5,574,142, 5,585,481, 5,587,371, 5,597,696 and 5,958,773. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Thus, the oligonucleotide may include other appended groups such as peptides, and may include hybridization-triggered cleavage agents (Krol et al., *BioTechniques* 6:958-976, 1988) or intercalating agents (Zon, *Pharm. Res.* 5:539-549, 1988). To this end, the oligonucleotide may be conjugated to another molecule, e.g., a chromophore, fluorophor, peptide, hybridization-triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

The oligonucleotide may also comprise at least one art-recognized modified sugar and/or base moiety, or may comprise a modified backbone or non-natural internucleoside linkage.

The oligonucleotides or oligomers according to particular embodiments of the present invention are typically used in 'sets,' which contain at least one oligomer for analysis of each of the CpG dinucleotides of genomic sequence SEQ ID NO: 1 to SEQ ID NO: 15 and sequences complementary thereto, or to the corresponding CpG, TpG or CpA dinucleotide within a sequence of the pretreated nucleic acids according to SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto. However, it is anticipated that for economic or other factors it may be preferable to analyze a limited selection of the CpG dinucleotides within said sequences, and the content of the set of oligonucleotides is altered accordingly.

Therefore, in particular embodiments, the present invention provides a set of at least two (2) (oligonucleotides and/or PNA-oligomers) useful for detecting the cytosine methylation state in pretreated genomic DNA (SEQ ID NO: 16 to SEQ ID NO: 75), or in genomic DNA (SEQ ID NO: 1 to SEQ ID NO: 15 and sequences complementary thereto). These probes enable diagnosis, classification and/or therapy of genetic and epigenetic parameters of colon cell proliferative disorders.

The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in pretreated genomic DNA (SEQ ID NO: 16 to SEQ ID NO: 75), or in genomic DNA (SEQ ID NO: 1 to SEQ ID NO: 15 and sequences complementary thereto).

In preferred embodiments, at least one, and more preferably all members of a set of oligonucleotides is bound to a solid phase.

In further embodiments, the present invention provides a set of at least two (2) oligonucleotides that are used as 'primer' oligonucleotides for amplifying DNA sequences of one of SEQ ID NO: 1 to SEQ ID NO: 75 and sequences complementary thereto, or segments thereof.

It is anticipated that the oligonucleotides may constitute all or part of an "array" or "DNA chip" (*i.e.*, an arrangement of different oligonucleotides and/or PNA-oligomers bound to a solid phase). Such an array of different oligonucleotide- and/or PNA-oligomer sequences can be characterized, for example, in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid-phase surface may be composed of silicon, glass, polystyrene, aluminum, steel, iron, copper, nickel, silver, or gold. Nitrocellulose as well as plastics such as nylon, which can exist in the form of pellets or also as resin matrices, may also be used. An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (*Nature Genetics Supplement*, Volume 21, January 1999, and from the literature cited therein). Fluorescently labeled probes are often used for the scanning of immobilized DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridized probes may be carried out, for example, via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

It is particularly preferred that the oligomers according to the invention are utilised for at least one of: detection of; detection and differentiation between or among subclasses of; diagnosis of; prognosis of; treatment of; monitoring of; and treatment and monitoring of colon cell proliferative disorders. This is enabled by use of said sets

for the detection or detection and differentiation of one or more of the following classes of tissues: colorectal carcinoma, colon adenoma, inflammatory colon tissue, grade 2 dysplasia colon adenomas less than 1 cm, grade 3 dysplasia colon adenomas larger than 1 cm, normal colon tissue, non-colon healthy tissue and non-colon cancer tissue.

The present invention further provides a method for ascertaining genetic and/or epigenetic parameters of the genomic sequences according to SEQ ID NO: 1 to SEQ ID NO: 15 within a subject by analyzing cytosine methylation and single nucleotide polymorphisms.

Wherein the object of the analysis is the detection or monitoring of colon cell proliferative disorders it is preferred that the methylation of one or more genomic sequences selected from Table 1 and/or their regulatory sequences are analysed. In this embodiment of the invention it is particularly preferred that the methylation of one or more of the sequences of the group consisting SEQ ID NO: 1 to SEQ ID NO: 15 and/or their regulatory sequences are analysed.

In a preferred embodiment the method comprises contacting a nucleic acid comprising one or more of SEQ ID NO: 1 to SEQ ID NO: 15 in a biological sample obtained from said subject with at least one reagent or a series of reagents, wherein said reagent or series of reagents, distinguishes between methylated and non-methylated CpG dinucleotides within the target nucleic acid.

Preferably, said method comprises the following steps: In the *first step*, a sample of the tissue to be analysed is obtained. The source may be any suitable source, such as cell lines, histological slides, biopsies, tissue embedded in paraffin, bodily fluids, ejaculate, urine, blood and all possible combinations thereof. Genomic DNA is then isolated from said biological sample, this may be by any means standard in the art, including the use of commercially available kits. Briefly, wherein the DNA of interest is encapsulated in by a cellular membrane the biological sample must be disrupted and lysed by enzymatic, chemical or mechanical means. The DNA solution may then be cleared of proteins and other contaminants e.g. by

digestion with proteinase K. The genomic DNA is then recovered from the solution. This may be carried out by means of a variety of methods including salting out, organic extraction or binding of the DNA to a solid phase support. The choice of method will be affected by several factors including time, expense and required quantity of DNA. Once the nucleic acids have been extracted, the genomic double stranded DNA is used in the analysis.

In the *second step* of the method, the genomic DNA sample is treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or another base which is dissimilar to cytosine in terms of hybridization behavior. This will be understood as 'pretreatment' herein.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to cytosine in terms of base pairing behavior.

In the *third step* of the method, fragments of the pretreated DNA are amplified, using sets of primer oligonucleotides according to the present invention, and an amplification enzyme. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel. Typically, the amplification is carried out using a polymerase chain reaction (PCR). The set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary, identical, or hybridize under stringent or highly stringent conditions to an at least 16-base-pair long segment of the base sequences of one or more of SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto.

In an alternative embodiment of the method, the methylation status of preselected CpG positions within the nucleic acid sequences comprising one or more of SEQ ID NO: 1 to SEQ ID NO: 15 may be detected by use of methylation-specific primer oligonucleotides. This technique (MSP) has been described in United States Patent No. 6,265,171 to Herman. The use of methylation status specific primers for the

amplification of bisulfite treated DNA allows the differentiation between methylated and unmethylated nucleic acids. MSP primers pairs contain at least one primer which hybridizes to a bisulfite treated CpG dinucleotide. Therefore, the sequence of said primers comprises at least one CpG dinucleotide. MSP primers specific for non-methylated DNA contain a "T" at the 3' position of the C position in the CpG. Preferably, therefore, the base sequence of said primers is required to comprise a sequence having a length of at least 9 nucleotides which hybridizes to a pretreated nucleic acid sequence according to one of SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto, wherein the base sequence of said oligomers comprises at least one CpG dinucleotide.

A further preferred embodiment of the method comprises the use of blocker oligonucleotides. The use of such blocker oligonucleotides has been described by Yu et al., *BioTechniques* 23:714-720, 1997. Blocking probe oligonucleotides are hybridized to the bisulfite treated nucleic acid concurrently with the PCR primers. PCR amplification of the nucleic acid is terminated at the 5' position of the blocking probe, such that amplification of a nucleic acid is suppressed where the complementary sequence to the blocking probe is present. The probes may be designed to hybridize to the bisulfite treated nucleic acid in a methylation status specific manner. For example, for detection of methylated nucleic acids within a population of unmethylated nucleic acids, suppression of the amplification of nucleic acids which are unmethylated at the position in question would be carried out by the use of blocking probes comprising a 'CpA' or 'TpA' at the position in question, as opposed to a 'CpG' if the suppression of amplification of methylated nucleic acids is desired.

For PCR methods using blocker oligonucleotides, efficient disruption of polymerase-mediated amplification requires that blocker oligonucleotides not be elongated by the polymerase. Preferably, this is achieved through the use of blockers that are 3'-deoxyoligonucleotides, or oligonucleotides derivitized at the 3' position with other than a "free" hydroxyl group. For example, 3'-O-acetyl oligonucleotides are representative of a preferred class of blocker molecule.

Additionally, polymerase-mediated decomposition of the blocker oligonucleotides should be precluded. Preferably, such preclusion comprises either use of a polymerase lacking 5'-3' exonuclease activity, or use of modified blocker oligonucleotides having, for example, thioate bridges at the 5'-termini thereof that render the blocker molecule nuclease-resistant. Particular applications may not require such 5' modifications of the blocker. For example, if the blocker- and primer-binding sites overlap, thereby precluding binding of the primer (e.g., with excess blocker), degradation of the blocker oligonucleotide will be substantially precluded. This is because the polymerase will not extend the primer toward, and through (in the 5'-3' direction) the blocker—a process that normally results in degradation of the hybridized blocker oligonucleotide.

A particularly preferred blocker/PCR embodiment, for purposes of the present invention and as implemented herein, comprises the use of peptide nucleic acid (PNA) oligomers as blocking oligonucleotides. Such PNA blocker oligomers are ideally suited, because they are neither decomposed nor extended by the polymerase.

Preferably, therefore, the base sequence of said *blocking oligonucleotides* is required to comprise a sequence having a length of at least 9 nucleotides which hybridizes to a pretreated nucleic acid sequence according to one of SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto, wherein the base sequence of said oligonucleotides comprises at least one CpG, TpG or CpA dinucleotide.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. Where said labels are mass labels, it is preferred that the labeled amplicates have a single positive or negative net charge, allowing for better detectability in the mass spectrometer. The detection may be carried out and visualized by means of, e.g., matrix assisted laser desorption/ionization mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas & Hillenkamp, *Anal Chem.*, 60:2299-301, 1988). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapour phase in an unfragmented manner. The analyte is ionized by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones. MALDI-TOF spectrometry is well suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut & Beck, *Current Innovations and Future Trends*, 1:147-57, 1995). The sensitivity with respect to nucleic acid analysis is approximately 100-times less than for peptides, and decreases disproportionally with increasing fragment size. Moreover, for nucleic acids having a multiply negatively charged backbone, the ionization process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallisation. There are now several responsive matrixes for DNA, however, the difference in sensitivity between peptides and nucleic acids has not been reduced. This difference in sensitivity can be reduced, however, by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. For example, phosphorothioate nucleic acids, in which the usual phosphates of the backbone are substituted with thiophosphates, can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut & Beck, *Nucleic Acids Res.* 23: 1367-73, 1995). The coupling of a charge tag to this modified DNA results in an increase in MALDI-TOF sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities, which makes the detection of unmodified substrates considerably more difficult.

In the fourth step of the method, the amplificates obtained during the third step of the method are analysed in

order to ascertain the methylation status of the CpG dinucleotides prior to the treatment.

In embodiments where the amplicates were obtained by means of MSP amplification, the presence or absence of an amplicate is in itself indicative of the methylation state of the CpG positions covered by the primer, according to the base sequences of said primer.

Amplicates obtained by means of both standard and methylation specific PCR may be further analyzed by means of hybridization-based methods such as, but not limited to, array technology and probe based technologies as well as by means of techniques such as sequencing and template directed extension.

In one embodiment of the method, the amplicates synthesised in *step three* are subsequently hybridized to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridization takes place in the following manner: the set of probes used during the hybridization is preferably composed of at least 2 oligonucleotides or PNA-oligomers; in the process, the amplicates serve as probes which hybridize to oligonucleotides previously bonded to a solid phase; the non-hybridized fragments are subsequently removed; said oligonucleotides contain at least one base sequence having a length of at least 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the present Sequence Listing; and the segment comprises at least one CpG , TpG or CpA dinucleotide.

In a preferred embodiment, said dinucleotide is present in the central third of the oligomer. For example, wherein the oligomer comprises one CpG dinucleotide, said dinucleotide is preferably the fifth to ninth nucleotide from the 5'-end of a 13-mer. One oligonucleotide exists for the analysis of each CpG dinucleotide within the sequence according to SEQ ID NO: 1 to SEQ ID NO: 15, and the equivalent positions within SEQ ID NO: 16 to SEQ ID NO: 75. Said oligonucleotides may also be present in the form of peptide nucleic acids. The non-hybridized amplicates are then removed. The hybridized amplicates are then detected. In this context, it is preferred that labels attached to the amplicates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

In yet a further embodiment of the method, the genomic methylation status of the CpG positions may be ascertained by means of oligonucleotide probes that are hybridised to the bisulfite treated DNA concurrently with the PCR amplification primers (wherein said primers may either be methylation specific or standard).

A particularly preferred embodiment of this method is the use of fluorescence-based Real Time Quantitative PCR (Heid et al., *Genome Res.* 6:986-994, 1996; also see United States Patent No. 6,331,393) employing a dual-labeled fluorescent oligonucleotide probe (TaqMan™ PCR, using an ABI Prism 7700 Sequence Detection System, Perkin Elmer Applied Biosystems, Foster City, California). The TaqMan™ PCR reaction employs the use of a nonextendible interrogating oligonucleotide, called a TaqMan™ probe, which, in preferred imbodiments, is designed to hybridize to a GpC-rich sequence located between the forward and reverse amplification primers. The TaqMan™ probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (e.g., phosphoramidites) attached to the nucleotides of the TaqMan™ oligonucleotide. For analysis of methylation within nucleic acids subsequent to bisulfite treatment, it is required that the probe be methylation specific, as described in United States Patent No. 6,331,393, (hereby incorporated by reference in its entirety) also known as the MethyLight™ assay. Variations on the TaqMan™ detection methodology that are also suitable for use with the described invention include the use of dual-probe technology (Lightcycler™) or fluorescent amplification primers (Sunrise™ technology). Both these techniques may be adapted in a manner suitable for use with bisulfite treated DNA, and moreover for methylation analysis within CpG dinucleotides.

A further suitable method for the use of probe oligonucleotides for the assessment of methylation by analysis of bisulfite treated nucleic acids In a further preferred embodiment of the method, the *fifth step* of the method comprises the use of template-directed oligonucleotide extension, such as MS-SNuPE as described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997.

In yet a further embodiment of the method, the *fifth step* of the method comprises sequencing and subsequent sequence analysis of the amplificate generated in the *third step* of the method (Sanger F., et al., *Proc Natl Acad Sci USA* 74:5463-5467, 1977).

Best mode

In the most preferred embodiment of the method the nucleic acids according to SEQ ID NO: 1 to SEQ ID NO: 15 are isolated and treated according to the first three steps of the method outlined above, namely:

- a. obtaining, from a subject, a biological sample having subject genomic DNA;
- b. extracting or otherwise isolating the genomic DNA;
- c. treating the genomic DNA of b), or a fragment thereof, with one or more reagents to convert cytosine bases that are unmethylated in the 5-position thereof to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties;

and wherein the subsequent amplification of d) is carried out in a methylation specific manner, namely by use of methylation specific primers or *blocking oligonucleotides*, and further wherein the detection of the amplicates is carried out by means of a real-time detection probes, as described above.

Wherein the subsequent amplification of d) is carried out by means of methylation specific primers, as described above, said methylation specific primers comprise a sequence having a length of at least 9 nucleotides which hybridizes to a pretreated nucleic acid sequence according to one of SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto, wherein the base sequence of said oligomers comprises at least one CpG dinucleotide.

Step e) of the method, namely the detection of the specific amplicates indicative of the methylation status of one or more CpG positions according to SEQ ID NO: 1 to SEQ ID NO: 15 is carried out by means of real-time detection methods as described above.

In an alternative most preferred embodiment of the method the subsequent amplification of d) is carried out in the presence of *blocking oligonucleotides*, as described above. Said *blocking*

oligonucleotides comprising a sequence having a length of at least 9 nucleotides which hybridizes to a pretreated nucleic acid sequence according to one of SEQ ID NO: 16 to SEQ ID NO: 75 and sequences complementary thereto, wherein the base sequence of said oligomers comprises at least one CpG, TpG or CpA dinucleotide.

Step e) of the method, namely the detection of the specific amplicates indicative of the methylation status of one or more CpG positions according to SEQ ID NO: 1 to SEQ ID NO: 15 is carried out by means of real-time detection methods as described above.

Additional embodiments of the invention provide a method for the analysis of the methylation status of genomic DNA according to the invention (SEQ ID NO: 1 to SEQ ID NO: 15, and complements thereof) without the need for pretreatment.

In the *first step* of such additional embodiments, the genomic DNA sample is isolated from tissue or cellular sources. Preferably, such sources include cell lines, histological slides, body fluids, or tissue embedded in paraffin. In the *second step*, the genomic DNA is extracted. Genomic DNA is then isolated from said biological sample, this may be by any means standard in the art, including the use of commercially available kits. Briefly, wherein the DNA of interest is encapsulated in by a cellular membrane the biological sample must be disrupted and lysed by enzymatic, chemical or mechanical means. The DNA solution may then be cleared of proteins and other contaminants e.g. by digestion with proteinase K. The genomic DNA is then recovered from the solution. This may be carried out by means of a variety of methods including salting out, organic extraction or binding of the DNA to a solid phase support. The choice of method will be affected by several factors including time, expense and required quantity of DNA.

In a preferred embodiment, the DNA may be cleaved prior to the treatment, and this may be by any means standard in the state of the art, in particular with methylation-sensitive restriction endonucleases.

In the *third step*, the DNA is then digested with one or more methylation sensitive restriction enzymes. The digestion is carried out such that hydrolysis of the DNA at the restriction site is informative of the methylation status of a specific CpG dinucleotide.

In the *fourth step*, which is optional but a preferred embodiment, the restriction fragments are amplified. This is preferably carried out using a polymerase chain reaction, and said amplificates may carry suitable detectable labels as discussed above, namely fluorophore labels, radionuclides and mass labels.

In the *fifth step* the amplificates are detected. The detection may be by any means standard in the art, for example, but not limited to, gel electrophoresis analysis, hybridization analysis, incorporation of detectable tags within the PCR products, DNA array analysis, MALDI or ESI analysis.

In the final step of the method the presence, absence or subclass of colon cell proliferative disorder is deduced based upon the methylation state of at least one CpG dinucleotide sequence of SEQ ID NO: 1 to SEQ ID NO: 15, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences of SEQ ID NO: 1 to SEQ ID NO: 15.

Monitoring, Diagnostic and/or Prognostic Assays for colon cell proliferative disorders

The present invention enables monitoring, diagnosis and/or prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within one or more of SEQ ID NO: 1 to SEQ ID NO: 15 may be used as markers. Said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a diagnosis and/or prognosis of events which are disadvantageous to patients or individuals.

Specifically, the present invention provides for monitoring, diagnostic and/or prognostic cancer assays based on measurement of differential methylation of one or more CpG dinucleotide sequences of SEQ ID NO: 1 to SEQ ID NO: 15, or of

subregions thereof that comprise such a CpG dinucleotide sequence. Typically, such assays involve obtaining a tissue sample from a test tissue, performing an assay to measure the methylation status of at least one of one or more CpG dinucleotide sequences of SEQ ID NO: 1 to SEQ ID NO: 15 derived from the tissue sample, relative to a control sample, or a known standard and making a diagnosis or prognosis based thereon.

In particular preferred embodiments, inventive oligomers are used to assess the CpG dinucleotide methylation status, such as those based on SEQ ID NO: 1 to SEQ ID NO: 75, or arrays thereof, as well as in kits based thereon and useful for the diagnosis and/or prognosis of colon cell proliferative disorders.

Kits

Moreover, an additional aspect of the present invention is a kit comprising, for example: a bisulfite-containing reagent; a set of primer oligonucleotides containing at least two oligonucleotides whose sequences in each case correspond, are complementary, or hybridize under stringent or highly stringent conditions to a 16-base long segment of the sequences SEQ ID NO: 1 to SEQ ID NO: 75; oligonucleotides and/or PNA-oligomers; as well as instructions for carrying out and evaluating the described method. In a further preferred embodiment, said kit may further comprise standard reagents for performing a CpG position-specific methylation analysis, wherein said analysis comprises one or more of the following techniques: MS-SNuPE, MSP, MethyLight™, HeavyMethyl™, COBRA, and nucleic acid sequencing. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

.Typical reagents (e.g., as might be found in a typical COBRA-based kit) for COBRA analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); restriction enzyme and appropriate buffer; gene-hybridization oligo; control hybridization oligo; kinase labeling kit for oligo probe; and radioactive nucleotides. Additionally, bisulfite conversion

reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kits (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (e.g., as might be found in a typical MethyLight®-based kit) for MethyLight® analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); LightCycler® and or TaqMan® probes; optimized PCR buffers and deoxynucleotides; and Taq polymerase.

Typical reagents (e.g., as might be found in a typical Ms-SNuPE-based kit) for Ms-SNuPE analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); optimized PCR buffers and deoxynucleotides; gel extraction kit; positive control primers; Ms-SNuPE primers for specific gene; reaction buffer (for the Ms-SNuPE reaction); and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kit (e.g., precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (e.g., as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene (or methylation-altered DNA sequence or CpG island), optimized PCR buffers and deoxynucleotides, and specific probes.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following example serves only to illustrate the invention and is not intended to limit the invention within the principles and scope of the broadest interpretations and equivalent configurations thereof.

Table 1

Gene/Genomic location	Genomic SEQ ID	Treated methylated SEQ IDs	Treated unmethylated SEQ IDs
-	1	16 & 17	46 & 47

BTF3 Homolog 1			
RING FINGER PROTEIN 4	2	18 & 19	48 & 49
TUMOR NECROSIS FACTOR RECEPTOR SUPERFAMILY MEMBER 16 PRECURSOR	3	20 & 21	50 & 51
Region of chromosome 14q31.1	4	22 & 23	52 & 53
Chondroitin sulfate proteoglycan 2 (versican) (CPSG2)	5	24 & 25	54 & 55
EYA 4	6	26 & 27	56 & 57
MOTHERS AGAINST DECAPENTAPLEGIC HOMOLOG 7 (SMAD 7)	7	28 & 29	58 & 59
ENDOPLASMIN PRECURSOR (94 KDA GLUCOSE- REGULATED PROTEIN) (GRP94) (GP96 HOMOLOG) (TUMOR REJECTION ANTIGEN 1)	8	30 & 31	60 & 61
Region of chromosome 6q25.1	9	32 & 33	62 & 63
B-CELL LYMPHOMA 6 PROTEIN (BCL-6) (ZINC FINGER PROTEIN 51) (LAZ-3 PROTEIN) (BCL- 5)	10	34 & 35	64 & 65
HOMEBOX PROTEIN ARISTALESS-LIKE 4	11	36 & 37	66 & 67
TRANSMEMBRANE PROTEIN WITH EGF-LIKE AND TWO FOLLISTATIN-LIKE DOMAINS 2	12	38 & 39	68 & 69
Hypothetical protein- leucine rich repeat	13	40 & 41	70 & 71
Candidate tumor suppressor 8p22	14	42 & 43	72 & 73
VESICULAR INHIBITORY AMINO ACID TRANSPORTER (GABA AND GLYCINE TRANSPORTER) (VESICULAR GABA TRANSPORTER) (HVIAAT)	15	44 & 45	74 & 75

EXAMPLES

MSP analysis of the genes according to Table 1.

In the following analysis the methylation status of the genes according to Table 1 were analysed by means of methylation specific amplification using the primers according to Table 2 (below).

The study was run on approximately 140 samples from colon, breast and liver carcinoma, normal tissue (peripheral blood lymphocytes, healthy colon and healthy tissue adjacent to colon carcinoma), colon polyps and other colon diseases. Genomic DNA was analyzed using the MSP technique after bisulfite conversion. Total genomic DNA of all samples was bisulfite treated converting unmethylated cytosines to uracil. Methylated cytosines remained conserved. Bisulfite treatment was performed with minor modifications according to the protocol described in Olek et al. (1996).

The sequence of interest was then amplified by means of methylation specific primers, the amplificate is then detected by means of methylation specific Taqman probes.

Table 2: Oligonucleotides for MSP Taqman.

Genomic SEQ ID NO:	Forward primer sequence	Probe	Reverse primer sequence
1	acggcggttttggttcgt SEQ ID NO: 76	cgacttaataacgacgtacgcgaaccc SEQ ID NO: 77	gtcatctaacaacgacctaactaacg SEQ ID NO: 78
2	ctctaaaccgcgaaaactccg SEQ ID NO: 79	cgcgttttggttcgggttcggg SEQ ID NO: 80	gggatgagaggttggttgacgttc SEQ ID NO: 81
3	agggcggttttggttgccg SEQ ID NO: 82	atcggcggttttagcgtgcggg SEQ ID NO: 83	ctacaacctaaacgacgcgct SEQ ID NO: 84
4	gaggtttcgggttcgaggttc SEQ ID NO: 85	acgtcacaaaaaaaaaacccacgtaataataacgaa SEQ ID NO: 86	ctttactacaacgaaaacgccg SEQ ID NO: 87
5	gattaaaaaatttgtttttattcggtcg SEQ ID NO: 88	cgcggaagtagagtagcgccggcg SEQ ID NO: 89	tccgcttcgaaaacctcga SEQ ID NO: 90
6	caaaatccgtcgacgataaaac SEQ ID NO: 91	tcgggttgggggttcgtattcgg SEQ ID NO: 92	acgtagttataggttttgatagttttac SEQ ID NO: 93
7	gaacgcgaaaaaattaacatctcg SEQ ID NO: 94	tttcgtttttatcgtggtgggtatgttcgtgttta SEQ ID NO: 95	tttcgggattgttggttggtgttt SEQ ID NO: 96
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10	ccaaaaatacgcgaccacg SEQ ID NO: 103	ctaaaccactaatattcgacgtaattctccgtattttac SEQ ID NO: 104	cgttttttcgtatttcggaagac SEQ ID NO: 105
11	tacgtgtgaggggttcgc SEQ ID NO: 106	ccaatcgaaactatccaaatcagataaccga SEQ ID NO: 107	ccaataaaaattcgtaaaaaaaacg SEQ ID NO: 108

12	cggttagtcggaggcgc SEQ ID NO: 109	cgaacgaaacaacctaaacgaaaaccccg SEQ ID NO: 110	ccaaaataaccaatcgacccg SEQ ID NO: 111
13	gggattaagattttcggttagtttcg SEQ ID NO: 112	caaacgaatcacataaaaaacgtaataacccgaa SEQ ID NO: 113	aacgctacgcgactaaattcga SEQ ID NO: 114
14	gggtaggggtgggtcgc SEQ ID NO: 115	cgtcggcggtgggcgatgtc SEQ ID NO: 116	gactcccgccccaacg SEQ ID NO: 117
15	cggaggggtacggagattacg SEQ ID NO: 118	cgaaaccctaaatatcccgaataacgccg SEQ ID NO: 119	cgacgacgcgcgaaa SEQ ID NO: 120

Reaction Conditions for Taqman PCR program:

Denaturation at 95°C for 10 minutes.

50 cycles: Denaturation at 95°C for 15 seconds.

Annealing at 62°C for 1 minute. Except SEQ ID NO:15, which has an annealing temp of 60°C.

Table 3: Results

Genomic SEQ ID NO	AUC	Sensitivity	Specificity
1	0.57	0.15	0.99
2	0.54	0.91	0.09
3	0.82	0.74	0.90
4	0.83	0.69	0.94
5	0.92	0.83	0.96
6	0.83	0.71	0.94
7	0.54	0.25	0.91
8	0.6	0.11	0.99
9	0.59	0.1	0.99
10	0.62	0.11	0.98
11	0.78	0.74	0.95
12	0.88	0.84	0.94
13	0.73	0.79	0.38
14	0.79	0.64	0.76
15	0.77	0.78	0.44

The following table gives the sensitivities and specificities of MSP assays analysing two genes.

Table 4 : Sensitivity and specificity of two member gene panel assays.

Genomic SEQ ID NOs.:	Sensitivity (%)	Specificity (%)
----------------------	-----------------	-----------------

9 & 12	88	97
11 & 12	88	97
12 & 15	90	95
6 & 7	87	97
5 & 11	89	94
6 & 12	84	94
12 & 3	80	98

HeavyMethyl analysis of the genes according to SEQ ID Nos: 3, 4 & 12.

In the following, methylation of the genomic sequences according to SEQ ID Nos: 3, 4 & 12 was analysed in 15 colorectal cancer samples (referred to as 'colon' in Table 5), and eight peripheral blood lymphocyte samples referred to as 'PBL' in Table 5).

Total genomic DNA of all samples was bisulfite treated converting unmethylated cytosines to uracil. Methylated cytosines remained conserved. Bisulfite treatment was performed with minor modifications according to the protocol described in Olek et al. (1996).

The sequence of interest was then amplified by means of primers specific for bisulfite treated DNA and a blocking oligonucleotide (in order to suppress amplification of non-methylated DNA). The 3'OH terminus of the blocking oligonucleotide was "capped" by a phosphate group to prevent polymerase from extending the primer during PCR reaction. The amplificate is then detected by means of methylation specific LightCycler probes, wherein one probe was labelled with 5' red640 and had a capped 3'OH terminus and the other was labelled with 3' Fluorescein.

Genomic SEQ ID NO:12

Primer SEQ ID NOS:121 & 122

Blocking oligonucleotide SEQ ID NOS: 123

Probe SEQ ID NOS: 124 (Fluorescein) & 125 (red640)

Genomic SEQ ID NO:3

Primer SEQ ID NOS: 126 & 127

Blocking oligonucleotide SEQ ID NOS: 128

Probe SEQ ID NOS: 129 (Fluorescein) & 130 (red640)

Genomic SEQ ID NO:4

Primer SEQ ID NOS: 131 & 132

Blocking oligonucleotide SEQ ID NOS: 133

Probe SEQ ID NOS: 134 (Fluorescein) & 135 (red640)

The reactions were performed in a total volume of 20 μ l using a LightCycler device (Roche Diagnostics). The real time PCR reaction mix contained 10 μ l of template DNA , 2 μ l of FastStart LightCycler reaction mix for hybridization probes (Roche Diagnostics, Penzberg),

Table 5: Results of HeavyMethyl assays

issue	Crossing points		Crossing points		Calculated Concentration DNA undiluted (pg/10 μ l)
	Genomic SEQ ID NO: 3	Genomic SEQ ID NO: 12	Genomic SEQ ID NO: 4 (undiluted)	Genomic SEQ ID NO: 4 (diluted)	
olon	36,73	38,22	39,58		5,85
olon	33,1	>51			386,
olon	39,8	42,84			57,
olon	39,74	38,75	32,54	33,89	482,
olon		>51			132,5
olon		38,82			29,95
olon	32,08	33,9	33,43	35,43	81
olon	37,03	36,91			284,3
olon	38,07	46,54			
olon	35,22	37,81	36,8	37,03	298,3
olon	33,66	36,19	33,23	34,01	135
olon	36,78	37,46	40,71		87
olon		37,23			57,
olon					166
olon	34,88	34,71	34,21	34,49	880,
BL					716,
BL					79
BL					584,
BL	38,55				181
BL					547
BL		49,04			76
BL					62
BL		39,75			983
100pg	36,34	37,29	34,95	34,51	583,
100pg	36,06	37,86	34,64	34,09	428,
MRoche 100ng		34,48			
MPhi 100ng					
ater					

Bold type figures indicate those classified as positive because the amplification curves were flat.

Serum analysis of the genes according to SEQ ID Nos: 3, 6, 14 & 12.

The assays as described above were then applied to a set of serum samples consisting 32 colorectal carcinoma serum samples, and compared to a set consisting of 54 normal serum samples, 11 serum samples from benign colon lesions and 6 serum samples from inflammatory colon disease. 130 ul of serum was used per assay and the results were quantitatively reported as positive or negative.

Table 6

Marker or combination of markers	Sensitivity	Specificity
12 (HeavyMethyl)	25	92.9
12 (MSP)	40.6	94.3
6 (MSP)	21.9	97.1
14 (MSP)	37.5	77.6
3 (MSP)	15.6	98.3
12, 3 & 6	50	95.7
12, 6 & 14	59.4	78.9

We claim:

1. A method for detecting, or for detecting and distinguishing between or among colon cell proliferative disorders in a subject, comprising contacting genomic DNA obtained from the subject with at least one reagent, or series of reagents that distinguishes between methylated and non-methylated CpG dinucleotides within at least one target region of the genomic DNA, wherein the target region comprises, or hybridizes under stringent conditions to at least 16 contiguous nucleotides of a sequence selected from the group consisting of SEQ ID NO: 1 to SEQ ID NO 15 respectively, wherein said contiguous nucleotides comprise at least one CpG dinucleotide sequence, and whereby detecting, or detecting and distinguishing between or among colon cell proliferative disorders is, at least in part, afforded.
2. A method according to claim 1 for detecting, or for detecting and distinguishing between or among colon cell proliferative disorders in a subject, comprising:
 - obtaining, from a subject, a biological sample having subject genomic DNA;
 - contacting the genomic DNA, or a fragment thereof, with one reagent or a plurality of reagents for distinguishing between methylated and non methylated CpG dinucleotide sequences within at least one target sequence of the genomic DNA, or fragment thereof, wherein the target sequence comprises, or hybridizes under stringent conditions to, at least 16 contiguous nucleotides of a sequence taken from the group consisting of SEQ ID NO: 1 to SEQ ID NO 15, said contiguous nucleotides comprising at least one CpG dinucleotide sequence; and
 - determining, based at least in part on said distinguishing, the methylation state of at least one target CpG dinucleotide sequence, or an average, or a value reflecting an average methylation state of a plurality of target CpG dinucleotide sequences, whereby detecting, or detecting and distinguishing between or among colon cell proliferative disorders is, at least in part, afforded.

3. The method of claims 1 and 2, wherein detecting, or detecting and distinguishing between or among colon cell proliferative disorders comprises detecting, or detecting and distinguishing between or among one or more tissues selected from the group consisting of colorectal carcinoma, colon adenoma, inflammatory colon tissue, grade 2 dysplasia colon adenomas less than 1 cm, grade 3 dysplasia colon adenomas larger than 1 cm, normal colon tissue, non-colon normal tissue, body fluids and non-colon cancer tissue.
4. The method of claim 2, wherein distinguishing between methylated and non methylated CpG dinucleotide sequences within the target sequence comprises converting unmethylated cytosine bases within the target sequence to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties.
5. The method of claim 2, wherein distinguishing between methylated and non methylated CpG dinucleotide sequences within the target sequence(s) comprises methylation state-dependent conversion or non-conversion of at least one CpG dinucleotide sequence to the corresponding converted or non-converted dinucleotide sequence within a sequence selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO 75, and contiguous regions thereof corresponding to the target sequence.
6. The method of claim 2, wherein distinguishing between methylated and non methylated CpG dinucleotide sequences within the target sequence comprises use of at least one nucleic acid molecule or peptide nucleic acid (PNA) molecule comprising, in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO 75, and complements thereof.

7. The method of claim 2, wherein the contiguous sequence comprises at least one CpG, TpG or CpA dinucleotide sequence.
8. A method according to claims 1 to 3 for detecting, or detecting and distinguishing between or among colon cell proliferative disorders in a subject, comprising:
 - a. obtaining, from a subject, a biological sample having subject genomic DNA;
 - b. extracting or otherwise isolating the genomic DNA;
 - c. treating the genomic DNA of b), or a fragment thereof, with one or more reagents to convert cytosine bases that are unmethylated in the 5-position thereof to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridization properties;
 - d. contacting the treated genomic DNA, or the treated fragment thereof, with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence of at least 9 nucleotides that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO 75, and complements thereof, wherein the treated genomic DNA or the fragment thereof is either amplified to produce at least one amplificate, or is not amplified; and
 - e) determining, based on a presence or absence of, or on a property of said amplificate, the methylation state of at least one CpG dinucleotide of a sequence selected from the group consisting SEQ ID NO: 1 to SEQ ID NO 15, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotides of a sequence selected from the groups consisting of SEQ ID NO: 1 to SEQ ID NO 15, whereby at least one of detecting, or detecting and distinguishing between colon cell proliferative disorders is, at least in part, afforded.

9. The method of claim 8, further comprising in step d) the use of at least one nucleic acid molecule or peptide nucleic acid molecule comprising in each case a contiguous sequence at least 9 nucleotides in length that is complementary to, or hybridizes under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NO: 16 to SEQ ID NO 75, and complements thereof, wherein said nucleic acid molecule or peptide nucleic acid molecule suppresses amplification of the nucleic acid to which it is hybridized.
10. The method of claim 8, wherein contacting or amplifying in d), comprises use of methylation-specific primers.

Abstract

The invention provides methods, nucleic acids and kits for detecting, or for detecting and distinguishing between or among colon cell proliferative disorders. The invention discloses genomic sequences the methylation patterns of which have utility for the improved detection of and differentiation between said class of disorders, thereby enabling the improved diagnosis and treatment of patients.

<110> Epigenomics AG

<120> Methods and nucleic acids for the improved detection of colon cell proliferative disorders.

<160> 135

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<213> Homo Sapiens

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<210> 18

<211> 2407

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 18

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<210> 19
 <211> 2407
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 19

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<210> 20
 <211> 3952
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 20

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<211> 3952

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 21

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<210> 22

<211> 2436

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 22

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<210> 23

<211> 2436

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 23

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<210> 24

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 24

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<210> 25
 <211> 2470
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 25

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<210> 26
 <211> 2470
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

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<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<211> 4721

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<223> chemically treated genomic DNA (Homo sapiens)

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<213> Artificial Sequence

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<211> 2494

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 31

<211> 2494

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 31

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<210> 33

<211> 2497

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 34

<211> 3095

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 35

<211> 3095

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 35

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<210> 36

<211> 2229

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 36

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<210> 37

<211> 2229

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 37

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2229

<210> 38

<211> 2820

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 38

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<210> 39

<211> 2820

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 39

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<210> 40

<211> 2280

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 40

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<210> 41

<211> 2280

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 41

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<210> 42
 <211> 2470
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 42

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 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 43

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<210> 46

<211> 3049

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 46

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<210> 47

<211> 3049

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 47

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<210> 48

<211> 2407

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 48

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<210> 49
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 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 49

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<210> 50
 <211> 3952
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> chemically treated genomic DNA (Homo sapiens)

<400> 50

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 <211> 3952
 <212> DNA
 <213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 51

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<211> 2436
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 52

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<210> 53
<211> 2436
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 53

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<210> 54

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 54

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<210> 55

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 55

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<210> 56

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 56

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<210> 57

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 57

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<210> 58

<211> 4721

<212> DNA

<213> Artificial Sequence

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<223> chemically treated genomic DNA (Homo sapiens)

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<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 59

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<210> 61
<211> 2494
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 61

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<210> 62

<211> 2497

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 62

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<210> 63

<211> 2497

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 63

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<210> 64

<211> 3095
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 64

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<210> 65
<211> 3095
<212> DNA
<213> Artificial Sequence

<220>
<223> chemically treated genomic DNA (Homo sapiens)

<400> 65

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<210> 66

<211> 2229

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 66

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<211> 2229

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 67

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<211> 2820

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 69

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<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 69

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<210> 70

<211> 2280

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 70

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<210> 71

<211> 2280

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 71

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<210> 72

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

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<210> 73

<211> 2470

<212> DNA

<213> Artificial Sequence

<220>

<223> chemically treated genomic DNA (Homo sapiens)

<400> 73

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<210> 74

<211> 5907

<212> DNA

<213> Artificial Sequence

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<223> chemically treated genomic DNA (Homo sapiens)

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